Virology 476 (2015) 100–105

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Brief Communication

High risk HPV E6 oncoproteins impair the subcellular distribution of the four and a half LIM-only protein 2 (FHL2)

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ARTICLE INFO

Article history:
Received 7 July 2014
Returned to author for revisions
31 July 2014
Accepted 21 November 2014
Available online 23 December 2014

Keywords:
HPV-E6 oncoprotein
FHL-2
Proteasome

ABSTRACT

HPVs are the causative agents of approximately 5% of all human cancers, with cervical cancer being the most predominant. To understand the mechanism of action of the viral E6 oncoprotein, we analysed the effects of E6 upon potential cellular target proteins. One candidate is FHL-2, involved in the regulation of signal transduction pathways from the multimeric complexes assembled at focal adhesions. We show that both HPV E6 and E6* can interact with FHL-2 *in vitro*, but unlike most E6 targets, FHL-2 does not appear to be an E6 degradation target. Analysis of the patterns of FHL-2 distribution within HPV-positive tumour-derived cells shows a significant alteration in the pattern of FHL-2 localisation when compared to non-HPV containing cells. This perturbation of FHL-2 distribution is proteasome-dependent and inhibition of E6 expression restores the normal distribution of FHL-2. These results confirm FHL-2 as a new interacting partner of the HPV-E6 oncoproteins.

Introduction

Human papilloma viruses (HPV) are responsible for 5% of the global cancer burden (Ferlay et al., 2014). Persistent HPV infection is associated with different types of cancer including, but not limited to, cervical cancer, and a subset of head and neck cancers in which the HPV genome is frequently found integrated into the genome of the host cell (zur Hausen, 1996). As a consequence of this DNA integration event, viral replicative capacity is lost and there is subsequent constitutive expression of the viral oncoproteins E6 and E7. Indeed, many studies have shown that both viral oncoproteins are essential for the continued proliferation and development of HPV positive tumours and the derived cell lines (von Knebel Doeberitz et al., 1992; Butz et al. 1996; Magaldi et al., 2012), highlighting their importance as potential targets for therapeutic intervention in HPV-induced malignancies.

Whilst E6 and E7 are continually expressed, alternative splicing within E6 results in the appearance of small truncated forms of E6, called E6* (Smotkin and Wettstein, 1986). Several studies have demonstrated that E6* isoforms function as potential regulators of full length E6 (Pim and Banks, 1999), although E6* also possesses a number of E6 independent functions (Filippova et al., 2007; Pim et al., 2009; Williams et al., 2014). Although E6 and E6* have been extensively studied, the mechanisms by which they contribute towards the development of malignancy still remain to be defined. One means of understanding how viral oncoproteins contribute towards malignant progression is to identify their respective cellular interactomes. In one such recent study, the Four and a Half LIM only protein 2 (FHL-2) was defined as a potential interacting partner of HPV-18 E6* (Rozenblatt-Rosen et al., 2012). FHL-2 is a multifunctional protein involved in a wide range of cellular processes, including the regulation of gene expression, cytoskeletal architecture, cell adhesion, cell survival, cell mobility and signal transduction (Li et al., 2001; Johannessen et al., (2006); Mori et al., 2006). This occurs primarily through the ability of FHL-2 to assemble multi-protein complexes via its LIM domains, which function as scaffolds to support the assembly of multimeric protein complexes (Kadrmas and Beckerle, 2004). This multi-functionality of FHL-2 appears to be dependent upon where it is localised within the cell. For example, cytoplasmic forms of FHL-2 interact with integrins and focal adhesion kinase (Gabriel et al., 2004), where it is involved in the regulation of signal transduction pathways originating at focal adhesions. In the nucleus, FHL-2 interacts with a number of DNA binding factors and can contribute to the control of a variety of different transcriptional programs, including those regulated by AP1 and -ß-catenin, and elevated levels of FHL-2 expression have been associated with increased tumour invasion and metastasis (Kleiber et al., 2007).

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http://dx.doi.org/10.1016/j.virol.2014.11.025
0042-6822/

However the role of FHL-2 in cancer development is controversial since, although it is up regulated in ovarian, skin, colon, lung and breast cancers, it is down regulated in prostate and rhabdomyosarcoma (Kleiber et al., 2007). To date there are no reports on the status of FHL-2 in cervical cancer, although FHL-2 is highly expressed in normal cervical tissue (Pontén et al., 2008).

Previous studies have shown that FHL-2 was an interacting partner of HPV-16 E7. As a consequence of this, the co-activator function of FHL-2 on the AP1-dependent and β-catenin-dependent promoters was impaired by E7 (Campo-Fernández et al., 2007). However, considering the recently published proteomic analyses indicating FHL-2 as a potential interacting partner of HPV-18 E6* (Rozenblatt-Rosen et al., 2012), we initiated a series of studies to investigate whether full-length HPV E6 or E6* has any propensity to modulate FHL-2 function. We present compelling evidence to suggest that HPV E6 specifically alters the subcellular distribution of FHL-2, which occurs in a proteasome-dependent manner.

Results

High risk HPV E6 proteins interact with FHL-2 in Vitro

Previous proteomic analyses indicated that FHL-2 was a specific target of HPV-18 E6* (Rozenblatt-Rosen et al., 2012). However since E6* shares a large amount of sequence homology with the N-terminus of the full-length HPV E6, we were interested in comparing the capacity of full-length E6 and E6* to interact with FHL-2 *in vitro*. To do this full length E6 protein and E6* from HPV-16 and HPV-18 were expressed as GST fusion proteins and purified, as was the low risk HPV-11 E6 for comparison. FHL-2 was *in vitro* transcribed, translated and radiolabeled with ^{35}S-Methionine and incubated with the different GST-E6 proteins at 4 °C. After several washes the protein complexes were analysed by SDS-PAGE and autoradiography. The results in Fig. 1A show a clear interaction between the different full-length E6 proteins from HPV-16 and HPV-18 with FHL-2, but somewhat weaker interaction with HPV-11 E6 and E6*. Since HPV-16 E7 has previously been shown to be an interacting partner of FHL-2 (Campo-Fernández et al., 2007), we also compared the relative abilities of E6 and E7 to interact with FHL-2 in an *in vitro* pull down assay. As can be seen from Fig. 1B, both HPV-16 and HPV-18 E6 interact with FHL-2 much more strongly than HPV-16 E7 under these assay conditions. These results are in agreement with recent proteomic studies, which also failed to detect FHL-2 in HPV E7 complexes (White et al., 2012; Rozenblatt-Rosen et al., 2012).

E6 affects FHL-2 localisation in HeLa cells

Several E6 interacting partners are targeted for proteasome-mediated degradation by the E6 oncoprotein (Banks et al., 2003; Beaudenon and Huibregtse, 2008). However we found little evidence that HPV-16 E6, HPV-18 E6, or the E6* isoforms, could affect the levels of FHL-2 expression in either *in vitro* degradation assays or in transient transfection experiments *in vivo* (data not shown). We were therefore interested in determining whether E6 could exert any potential effects on the pattern of FHL-2 expression in cells expressing endogenous E6. To do this we monitored the pattern of FHL-2 expression in HeLa cells by immunofluorescence following siRNA ablation of the expression of either E6 alone or E6/E7 in combination; siLuciferase was used as a control. The results in Fig. 2 show that the levels of FHL-2 expression in HeLa cells are quite low and confined primarily to the cell periphery. Following ablation of either E6 or E6/E7 expression, there is a dramatic increase in nuclear p53 levels as would be expected. Most interestingly, there is also a concomitant general increase in the strength of FHL-2 staining, with a marked accumulation of FHL-2 in fibrous structures within the cytoplasm in many of the p53-rescued cells. This pattern of FHL-2 distribution is very similar to what has been reported previously (Müller et al., 2002; Wixler et al., 2007).

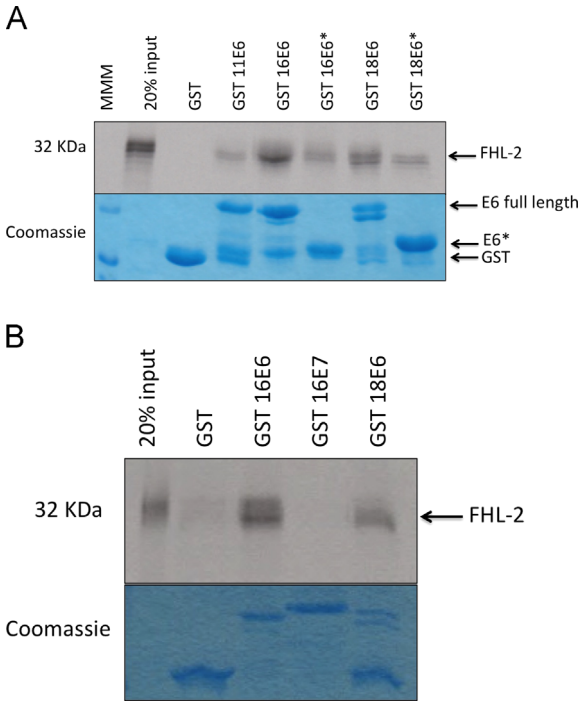

Fig. 1. FHL-2 interacts specifically with HPV E6 protein *in Vitro* and *in Vivo*. Purified GST-E6 (A) and GST-E7 (B) proteins were incubated with *in Vitro* translated FHL-2 protein at room temperature. After several washes the bound proteins were analysed by SDS-PAGE and autoradiography. The FHL-2 bound protein is indicated by the arrows and the lower panel shows the Coomassie stain indicating the GST protein balance. 20% input is also indicated.

E6 perturbs FHL-2 expression in a proteasome-dependent manner

FHL-2 interacts with integrins in the cytoplasm allowing multi-protein complex formation and thereby regulating signal transduction (Wixler et al., 2000). FHL-2 has also been demonstrated to be a key regulator of cell growth, cell fate determination, cell differentiation, and remodelling of the actin cytoskeleton (Li et al., 2001; Mori et al., 2006). The above results indicate that loss of E6/E7 expression triggers a re-distribution in the pattern of FHL-2 expression. To determine whether the proteasome has a role in the ability of E6 to perturb the pattern of FHL-2 expression, we repeated the immunofluorescence analysis in the presence of the proteasome inhibitor Z-Leu-Leu-Leu-Al (CBZ) including C33A, a HPV negative cell line, and two HPV containing cell lines CaSki (HPV16) and HeLa (HPV18). As can be seen in Fig. 3A and B, untreated HeLa and CaSki cells display low levels of expression of both FHL-2 and p53. However proteasome inhibition results in a significant increase in nuclear p53 and a dramatic increase in the levels of cytoplasmic fibrous structured FHL-2, both of which are similar to the results obtained following ablation of E6/E7 expression. In contrast the HPV negative C33A cells display a low level of expression of FHL-2 expression and mutant nuclear p53, and CBZ treatment has no effect on the pattern of p53 or FHL-2 staining (Fig. 3C). This indicates that E6/E7 can modulate the pattern of FHL-2 distribution in a manner that is proteasome-dependent.

To determine whether these FHL-2 cytoplasmic structures, which are restored following proteasome inhibition or E6 removal, are

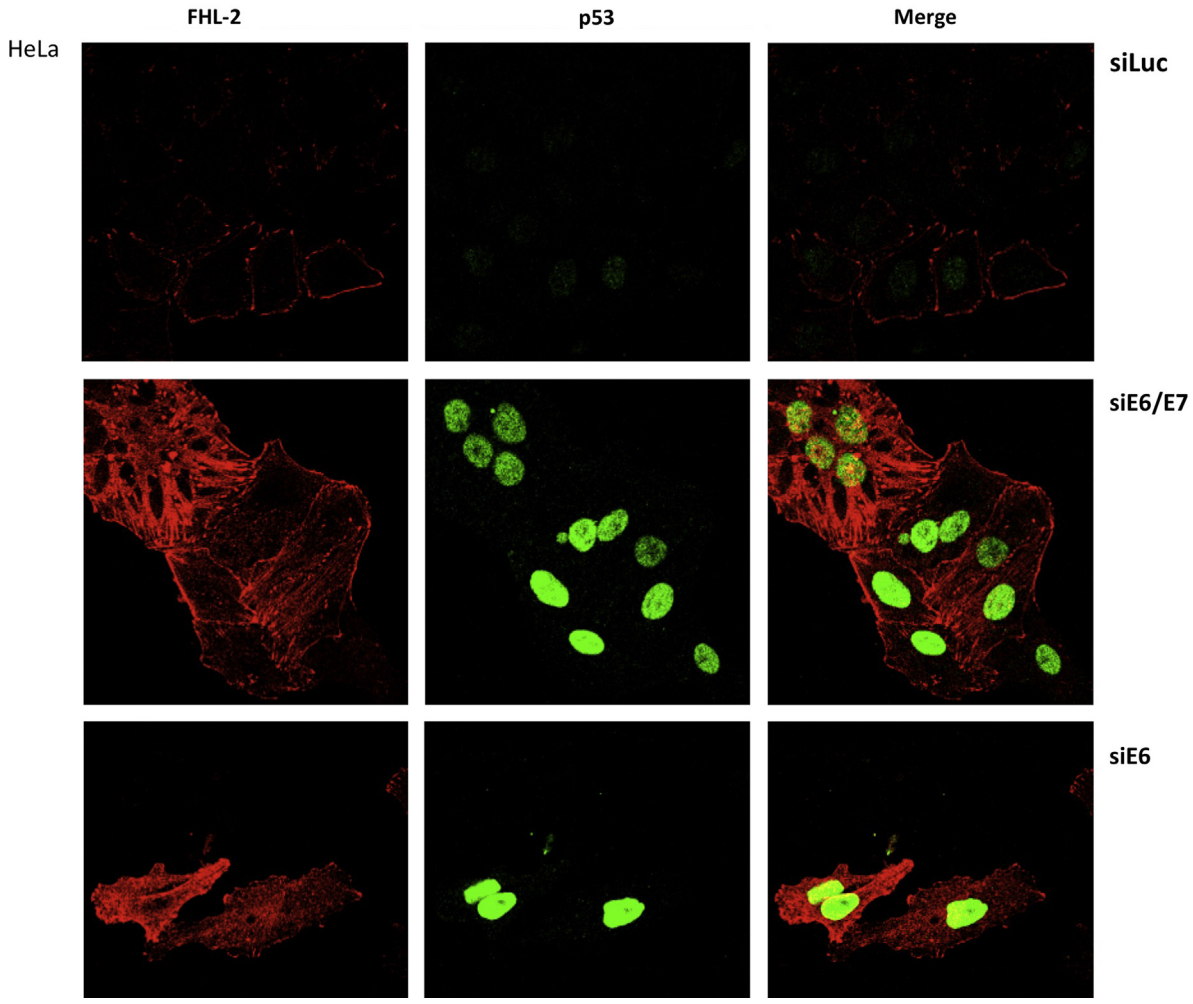

Fig. 2. E6 changes the pattern of FHL-2 expression in HeLa cells. HeLa cells were transfected with siRNA luciferase (si luc), siRNA E6/E7 or siRNA E6 and after 72 h postransfection cells were fixed and stained for FHL-2 and p53. Note that the p53 signal becomes stronger after E6/E7 and E6 ablation and that FHL-2 accumulates in the cytoplasm, with the appearance of fibrillar structures in many cases.

associated with stress fibres, we repeated the immunofluorescence analysis by co-staining for FHL-2 and actin stress fibres with Phalloidin following proteasome inhibition. As can be seen in Fig. 3D, FHL-2 is again rescued after proteasome inhibition and shows a significant degree of co-localisation with actin stress fibres. In contrast, the HPV negative cell line C33A did not show any change in FHL-2 distribution after proteasome inhibition (Fig. 3E) and actin stress fibre distribution remained unchanged.

Discussion

HPV induced carcinogenesis relies on the continued expression of E6 and E7 (Yoshinouchi et al., 2003). This, in turn, regulates a plethora of signal transduction pathways leading to the transformation and immortalisation of the cell.

The ability of E6 to induce the degradation of its cellular partners has been proposed to be one of the fundamental mechanisms by which E6 can contribute towards malignant progression (Howie et al., 2009). A recent protein-based analysis provided new insights into the E6 interacting partners (Rozenblatt-Rosen et al., 2012). Among them, FHL-2 is of particular interest since it regulates signal transduction by acting as a scaffold protein in an integrin-dependent manner (Li et al., 2001). In this study we show that high-risk HPV E6 proteins interact with and modulate the localisation of FHL-2.

The interaction of FHL-2 with high-risk E6 proteins was evident from the *in vitro* binding assays. Based on the previous studies (Rozenblatt-Rosen et al., 2012), an interaction between FHL-2 and HPV-18 E6* was to be expected. Nevertheless, we demonstrated that such interaction was somewhat stronger with the full-length forms of the high-risk HPV E6 proteins, although a significant degree of association was obtained with the low risk HPV-11 E6, suggesting that E6 association with FHL-2 might be important for the life cycles of diverse HPV types. Despite extensive attempts we did not detect any significant degree of interaction between HPV-16 E7 and FHL-2 (Campo-Fernández et al., 2007), although at this stage we cannot exclude that E7 may nonetheless contribute towards a modulation of FHL-2 activity in vivo. These biochemical observations are also in agreement with recent proteomic analyses, which also failed to detect FHL-2 in E7 complexes (White et al., 2012; Rozenblatt-Rosen et al., 2012).

Many of the interacting partners of the high-risk HPV E6 proteins are directed for degradation via the proteasome. However this does not seem to be the case with FHL-2, or at least not directly. Using either in vitro degradation assays or transient transfection assays we were unable to demonstrate that E6 could target FHL-2 for degradation. Likewise, no major changes in the levels of FHL-2 expression in western blotting following E6 ablation or proteasome inhibition in HPV positive tumour derived cell lines was observed. Nonetheless, in both CaSki and HeLa cells,

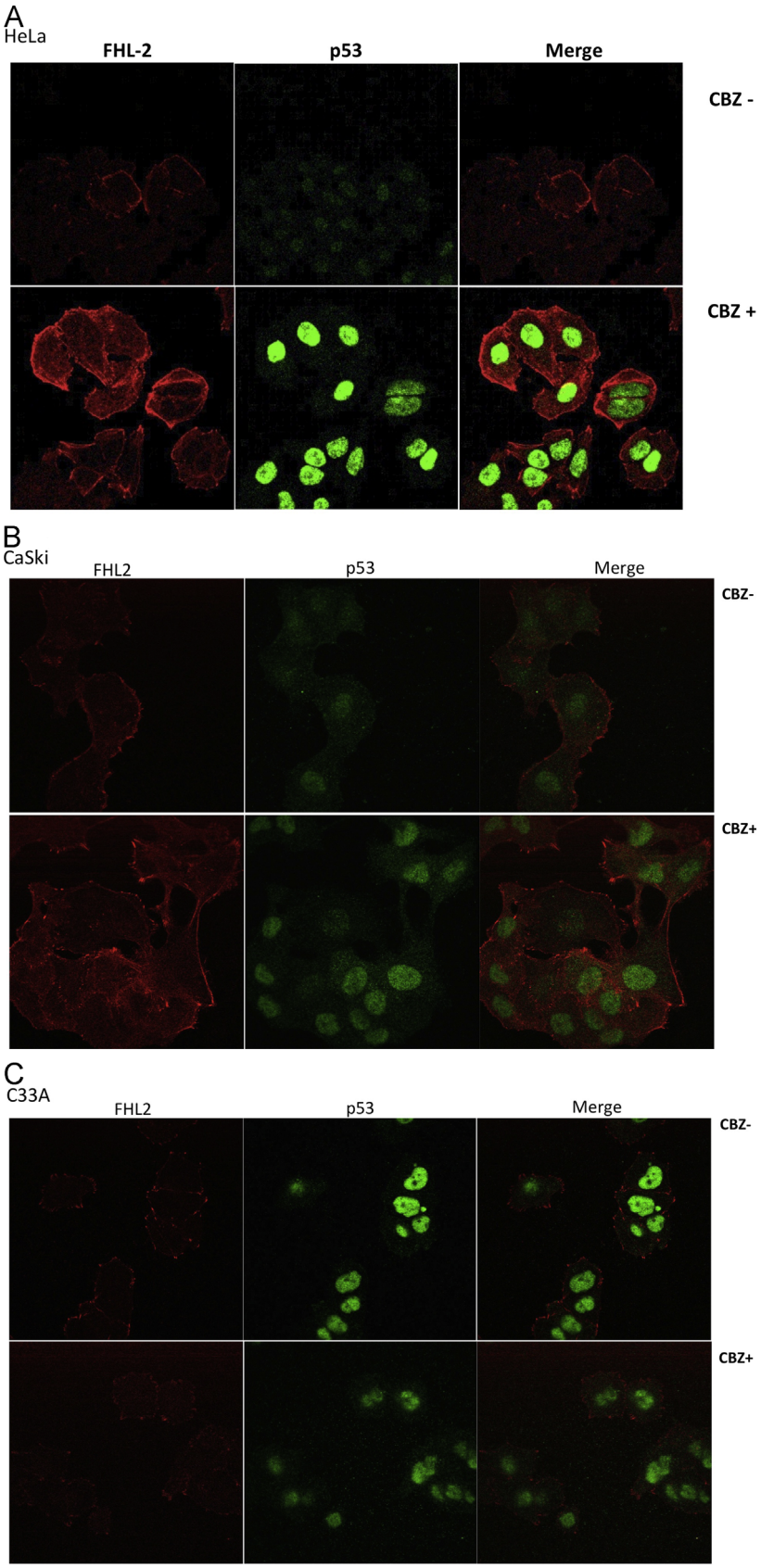

Fig. 3. FHL-2 localisation in HPV positive cells is regulated by the proteasome. C33A, Caski and HeLa cells were seeded on coverslips in six well plates and grown for 24 h to allow attachment. The next day cells were treated with proteasome inhibitor Z-Leu-Leu-Leu-Al (CBZ) (Sigma) for 3 h prior to fixing as indicated (+). Then cells were stained for FHL-2 and p53, with HeLa cells in Panel A, CasKi cells in Panel B and C33A cells in Panel C. Note the increased nuclear p53 in the HPV-positive CBZ treated cells and the concomitant increase in cytoplasmic FHL-2. Panel D, HeLa cells and Panel E, C33A cells were seeded on coverslips in six well plates and grown for 24 h to allow attachment. The next day cells were treated with proteasome inhibitor Z-Leu-Leu-Leu-Al (CBZ) (Sigma) 3hrs prior to fixing and stained for FHL-2 using anti-FHL-2 antibody and actin stress fibres detected using Phalloidin (Molecular Probes). Note that the CBZ treated HeLa cells show some co-localisation of FHL-2 with stress fibres which is not seen in C33A cells.

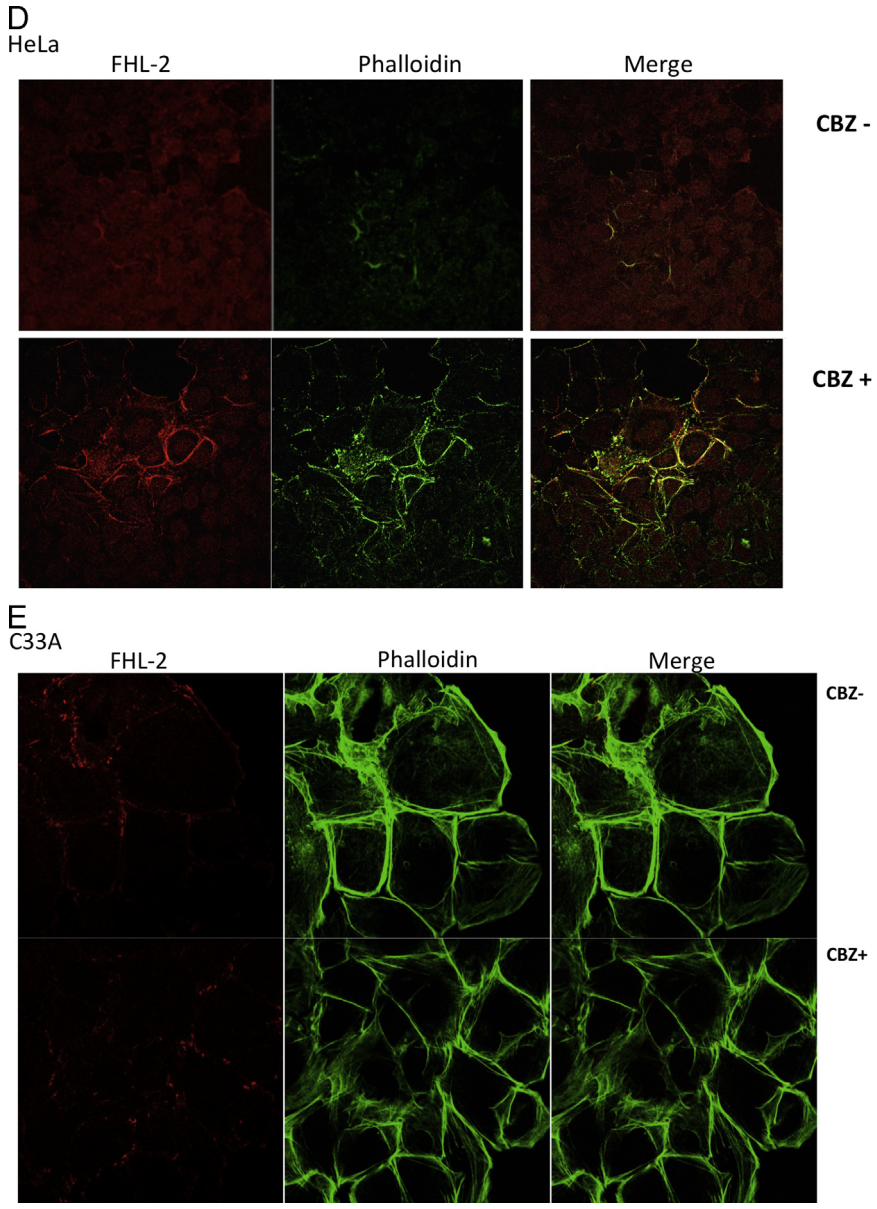

Fig. 3. (*continued*)

FHL-2 expression was quite weak, and was restricted to the cell periphery. However upon ablation of E6 or E6/E7 there was a dramatic accumulation of FHL-2, both in terms of the levels of signal intensity and its subcellular distribution. Likewise, treatment of CaSKi and HeLa cells with proteasome inhibitors produced similar changes in the pattern of FHL-2 expression, an effect that was not observed in HPV-negative C33A cells. Taken together these results suggest that E6 has a marked effect upon the distribution of FHL-2 within cells, and this is proteasome dependent. However, it is tempting to speculate that the apparent overall increase in FHL-2 levels is actually a consequence of FHL-2 accumulation at a defined location within the cell or, most likely, one of epitope re-exposure on FHL-2 following loss of E6, rather than an actual real alteration in FHL-2 total protein levels. Future studies will be required to investigate these aspects further.

Finally the use of phalloidin staining following proteasome inhibition in HPV positive cells, also demonstrated that some of the sites of FHL-2 accumulation are actin stress fibres, which contrasts with HPV-negative C33A cells where FHL-2 does not accumulate in such structures, despite there being very clear stress fibre structures. This is intriguing since stress fibres are involved in a large number of signal transduction processes and have essential roles in cell adhesion, integrin signalling and mechano-transduction and cell morphogenesis (Tojkander et al., 2012).

These results define FHL-2 as a novel interacting partner of the high risk HPV E6 oncoproteins, and indicate that the perturbation of FHL-2 subcellular distribution and the subsequent disruption of its normal role as a scaffold protein in diverse cell signalling pathways might be an important element in HPV-induced tumourigenesis but also in the HPV life cycle.

Materials and methods

Cells and transfections

C33A (HPV negative cells), CaSki and HeLa (HPV16 and 18 containing cells) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS).

GST-fusion proteins purification

GST-fusion proteins production and purification were described previously (Pim et al., 1997).

In vitro translation and In vitro binding assays

1 μg of FHL-2 expressing plasmid was *in vitro* transcribed and translated using the TNT T7 Coupled Reticulocyte Lysate System® (Promega) employing [^{35}S]-Methionine according to the manufacturer´s instructions. For the *in vitro* binding assays equal amounts of the different E6 GST-fusion proteins were incubated with *in vitro* translated FHL-2 in PBS at room temperature for 90 min. After extensive washing with 0.2% NP-40/PBS the bound proteins were analysed by SDS-PAGE and autoradiography. GST-fusion proteins amount was analysed by Coomassie staining.

Immunofluorescence and cell imaging

72 h after siRNA transfection HeLa cells were fixed in4%paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton-X100, the cells were then incubated for 2 h at 37 °C with polyclonal anti-FHL-2 antibody (Sigma Aldrich), monoclonal anti-p53 (DO1, Santa Cruz Biotechnology) either alone or in combination, washed extensively with PBS, and incubated with anti-rabbit or anti-mouse conjugated to rhodamine or fluorescein (Molecular Probes) at 37 °C. Slides were washed and mounted in Vectashield mounting medium (Vector Laboratories). CBZ treated HeLa cells were processed in the same manner 72hrs post siRNA transfection and were incubated with anti p53 (Santa Cruz), anti-FHL-2 antibody (Sigma Aldrich) or Phalloidin (Molecular Probes). Slides were analysed with a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480 and 510 nm. The data were collected with a 60X objective oil immersion lens.

siRNA silencing and fractionation experiments

For the delivery of the siRNA (Dharmacon), 120,000 HeLa cells were seeded in 6 cm diameter dishes and maintained for 24 h to allow attachment, then cells were transfected with siRNA against HPV-18 E6 (5´-CUCUGUGUAUGGAGACACATT) or HPV-18 E6/E7 (5′CAUUUACCAGCCCGACGAG) and Luciferase as a control, using the Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. 72 h after silencing, cells were harvested and protein extracted using the sub-cellular proteome extraction kit (Calbiochem) according to the manufacturer's instructions, protein levels were evaluated by western blot.

Acknowledgments.

This work was partially supported by the Associazione Italiana per la Ricerca sul Cancro and by CONACyT Mexico grant CB-166808. JMM was the recipient of an Arturo Falaschi Fellowship. We are very grateful to Miranda Thomas for valuable comments on the manuscript.

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